COMPARISON OF ESTERASE RELEASE FROM UNGERMINATED SPORES OF VIRULENT AND AVIRULENT ISOLATES OF *Chalara elegans* Nag Raj and Kendrick.

Synodalia Ch. Wattimena[1]

ABSTRACT

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Chalara elegans Nag Raj and Kendrick (syn. *Thielaviopsis basicola* (Berk. and Br.) Ferr.) is a fungal pathogen that causes black root rot disease on many plant species. The ability of virulent and avirulent isolates of *C. elegans* to release nonspecific esterase was studied. Virulent isolates (BK28, BC94PA, NC49A, HRB, AK208, CKP, AK89) released esterase after 45 minutes of contact with an artificial (plastic wrap) surface whereas avirulent isolates (BK28R, BK101, BK102, WASH, FL/W) did not released esterase after 45 minutes of contact with an artificial (plastic wrap) surface. This result suggests that esterase may be important in the penetration process of *C. elegans*. Low temperature (5°C) reduced the ability of a virulent isolate of *C. elegans* (BK28) to release esterase on plastic wrap. While growing on V8 juice agar, virulent isolates released significantly more esterase than avirulent isolates.

Keywords : Esterase, avirulent and virulent isolates, *Chalara elegans* Nag Raj and Kendrick

INTISARI

Wattimena, S.Ch. 2006. Perbandingan kemampuan menghasilkan enzim esterase antara isolat yang bersifat* virulent *dan* avirulent *Chalara elegans. Berkala Ilmiah Biologi 5 (1) : 23 - 31.

Chalara elegans Nag Raj dan Kendrick (syn. Thielaviopsis basicola (Berk. dan Br.) Ferr.) adalah jamur yang dapat menyebabkan penyakit 'black root' *pada banyak spesies tanaman. Kemampuan dari isolat-isolat* C. *elegans yang dapat menyebabkan penyakit* (virulent) *dan yang tidak dapat menyebabkan penyakit* (nonvirulent) *untuk menghasilkan/memproduksi enzim esterase diteliti. Isolat-isolat yang bersifat* virulent *(BK28, BC94PA, NC49A, HRB, AK208, CKP, AK89) memproduksi esterase setelah 45 menit kontak dengan permukaan* artificial (plastic wrap) *sedangkan isolat-isolat yang bersifat* nonvirulent *(BK28R, BK101, BK102, WASH, FL/W) tidak memproduksi esterase setelah 45 menit kontak dengan permukaan* artificial (plastic wrap). *Hal ini menunjukkan bahwa esterase mungkin memainkan peran yang penting dalam proses penetrasi dari C. elegans pada permukaan tanaman. Temperatur yang rendah (5°C) menurunkan kemampuan dari isolat* virulent *(BK28R) untuk memproduksi esterase pada permukaan* plastic wrap. *Pada saat tumbuh di medium V8 agar, isolat-isolat yang bersifat* virulent *memproduksi esterase jauh lebih banyak dibandingkan isolat-isolat yang bersifat* nonvirulent.

Kata kunci : Esterase, isolat virulent *dan* nonvirulent, *Chalara elegans* Nag Raj and Kendrick

INTRODUCTION

Many fungal pathogens are able to penetrate host plants directly through intact surfaces. For fungal pathogens that penetrate directly through intact plant surfaces, the plant cuticle is the first physical barrier to be overcome. The involvement of both mechanical force and enzymatic degradation of the plant cuticle during penetration has been proposed (Marks & Hine, 1988; McKeen, 1974). Cutinase, a serine esterase, is the enzyme suggested to assist penetration of cuticle (Dickman *et al.*, 1982; Kolattukudy, 1985; Koller *et al.*, 1982).

Chalara elegans Nag Raj and Kendrick (syn. *Thielaviopsis basicola* Berk. and Br.), a soil associated fungal plant pathogen, has been associated with black root rot disease of a broad range of plant species including cotton, carrot, bean, peanut, pansies and tobacco (Johnson & Doyle, 1986). Under laboratory conditions, *C. elegans* can infect both root and shoot tissues of beans. Virulent and avirulent isolates of *C. elegans* on bean root and shoot tissues have been recognized (Sualang, 1997). *C. elegans* thus provides a good model system for analysis of attributes that are associated with

[1] Jurusan Biologi, FMIPA, Universitas Pattimura, Ambon

fungal virulence. It has been reported that *C. elegans* can penetrate epidermal cells of root and hypocotyl of bean without forming appresoria (Christou, 1962). Sualang (1997) reported that an avirulent isolate of *C. elegans* failed to penetrate bean surfaces, and that 10-36 spores of a virulent isolate of *C. elegans* were needed to initiate infection at point inoculation sites on both roots and hypocotyls of bean seedlings. On this basis, Sualang suggested that spores of *C. elegans* may have to release sufficient amount of hydrolytic enzymes on the surface of beans in order to penetrate. The relative absence of appressoria during penetration by *C. elegans* also suggests that hydrolytic enzymes may play a more important role than mechanical force during penetration by this fungus.

The objectives of these studies were to find out (1) whether spores of virulent and avirulent isolates of *C. elegans* differ in their ability to release nonspecific esterase, (2) the time course of esterase release and (3) the effect of temperature on the release of esterase.

MATERIALS AND METHODS

Fungal cultures

Twelve isolates of *C. elegans*, provided by Dr. Z.K. Punja (Simon Fraser University, Canada) (Table 1.), were used in these studies. The isolates were grown in the dark on 10% V8 juice agar. Phialospore suspensions were prepared from 14-day old cultures, passed through sterile cheesecloth to remove mycelial fragments, and adjusted to desired concentrations in sterile distilled water.

Virulence tests

Virulence tests were conducted to confirm the differences in virulence of the 12 isolates of *C. elegans* on bean. Seeds of bean (*Phaseolus vulgaris* L.) cultivar Royal Burgundy that had been rinsed with distilled water were put between two sheets of moistened germination paper (Anchor Paper, St.Paul, MN) lying on a single sheet of wax paper. The papers were then rolled into cylinders (rag dolls) and placed vertically in an incubator at 28°C. Distilled water was added as required to keep the rag dolls moist. After 6 days selected seedlings were transferred to new rag dolls, so that each rag doll contained five bean seedlings of uniform size.

Spore suspensions from each of the 12 isolates were prepared from 14-day-old cultures and adjusted to $1x10^6$ spores/ml. Spore suspensions were sprayed onto 6–day-old bean seedlings lying on moist germination paper. After inoculation, the spore droplets on seedlings were allowed to become nearly dry before rolling the papers again into rag dolls. After that the rag dolls were kept in a growth room with 12 hours photoperiod and at 22-24°C. The rag dolls were kept moistened by adding distilled water. The interaction phenotypes were recorded 10 days after inoculation. Isolates that produced many big necrotic lesions on most parts of the bean seedlings were considered as virulent isolates, while isolates that produced only one small fleck or occasionally two small lesions on the bean seedlings were considered as avirulent isolates.

Esterase assays

Assays for non-specific esterase activity were done using *p*-nitrophenyl butyrate as substrate as described by Kunoh *et al.*, (1990). Reaction mixtures consisted of 1.5 ml of substrate (6×10^{-4} M) in phosphate buffer (0.05M, pH 7.5) and 1.5 ml of supernatant. The liberation of *p*-nitrophenol from *p*-nitrophenyl butyrate was measured spectrophotometrically at 405 nm. The reaction was carried out at 30°C for 10 minutes. The substrate was brought into solution by sonication and a blank reaction mixture consisting of 1.5 ml of substrate (6×10^{-4} M) in phosphate buffer (0.05M, pH 7.5) and 1.5 ml of distilled water was used as a control. The absorbance of the control was subtracted from that of the reaction mixture to correct for the autohydrolysis of the substrate that occurs in the absence of esterase.

Esterase assays for 12 isolates using unwashed spores

Spores of 12 isolates of *C. elegans* and their associated extracellular material were harvested from 14-day-old cultures and brought into suspension and adjusted to a final concentration of $2x10^6$ spores/ml. Spore suspensions (10 ml) were placed in centrifuge tubes and centrifuged for 6 minutes at 3000 g at room temperature. The supernatants were then taken for esterase test to determine whether spore extracellular materials contain esterase activity.

Time course of esterase production for 12 isolates using washed spores

Esterase activity present in extracellular substances in initial spore suspensions was removed by repetitive washing. Spore suspensions were placed in centrifuge tubes and centrifuged for 6 minutes at 3000 g. The supernatants were then taken for the esterase test immediately and the pellet was resuspended in sterile distilled water. The centrifugation procedure was repeated until no esterase activity was found in the supernatant. The washed spores were then adjusted to a final concentration of $2x10^6$ spores/ml and applied to the surface of sterile plastic wrap (11 cm x 11 cm) placed as an overlay on V8 juice agar in petri dishes (9 cm diameter). Each petri dish recieved 7 ml of spore suspension. The petri dishes were gently shaken to distribute the spores well on the plastic wrap that covered the entire agar surface in the petri dishes. The petri dishes with applied spores were then kept in the dark for 45 minutes at room temperature. After incubation, the plastic wrap with spore suspension was carefully taken from the petri dish and put into a centrifuge tube which already contained 15 ml phosphate buffer (0.05 M, pH 7.5) and 0.1 ml Triton X. The contents of the tube were mixed (Vortex) and then centrifuged for 6 minutes. The supernatant was assayed for esterase activity as described previously. As a control, esterase assay was conducted using washes of sterile plastic wrap that had been overlaid on V8 juice agar but not treated with spore suspension.

To identify the time required for washed spores to produce extracellular esterase activity, spores of four isolates (CKP, BK28, BK28R, BK101) of *C. elegans* were washed and applied to the plastic wrap as described above. The plastic wrap with applied spores was then incubated at room temperature. After the required incubation time on the plastic wrap, the spore suspension was assayed for esterase activity as described above.

To find out the effect of temperature on the release of esterase, plastic wraps with applied spores of two isolates (BK28 and BK28R) were incubated at 5°, 15°, 24°C.

Each experiment was repeated three times. Absorbances were converted to concentration of nitrophenol (µmol/ml) using a standard curve prepared from 0.005 to 0.6 µmol/ml range of pure *p*-nitrophenol.

Data analysis

One-way analysis of variance (ANOVA) test and Tukey post hoc means comparisons were used to analyze some data. Computer program ORIGIN 7 (Origin Lab Corporation) was used to process the data.

RESULTS

Virulence tests

Seven of the 12 isolates of *C. elegans* were virulent and five isolates were avirulent on beans (Table 1.). Interaction phenotypes on root and hypocotyl tissues were similar. Virulent isolates produced many necrotic lesions on hypocotyls and roots of bean seedlings whereas avirulent isolates produced only one small fleck or occasionally two small lesions (Figure 1.). The color of lesions was dark brown to black.

Esterase assays for 12 isolates using unwashed spores

Esterase activity in supernatants of unwashed spores of 12 isolates, and in successive washes of spores by centrifugation for six of these 12 isolates of *C. elegans* are shown in Figure 2. and Table 2., respectively. Virulent isolates released much more esterase than avirulent isolates while growing in V8 agar. Spores of virulent isolates were completely free of esterase activity after the fourth or fifth wash whereas spores of avirulent isolates only needed to be washed once to remove esterase activity (Table 2.). Based on these data, spores used for all subsequent assays were washed five times before incubating them on the surface of sterile plastic wrap over V8 agar.

Esterase assays for 12 ilsolates using washed spores

Production of esterase activity by washed spores was correlated with virulence of *C. elegans* on bean (Figure 3.). Washed spores of all of the seven virulent isolates released esterase during 45 minutes of incubation on the surface of plastic wraps, whereas no esterase was released by spores of the five avirulent isolates.

Time required for washed spores to produce extracellular esterase activity

Washed spores of virulent isolates of

Table 1. Isolates of *Chalara elegans* used in these studies, their geographic, and host origins, their double stranded RNAs (dsRNAs) status and their virulence on bean (*Phaseolus vulgaris* L.)

Isolate	Geographic Origin*	Host* origin	dsRNA*	Virulence on bean
BK28	Kings County, CA	Cotton	+	Virulent
BC94PA			unknown	Virulent
NC49A	Buncombe, NC	Tobacco	+	Virulent
HRB	Cloverdale, BC	Motherwort	+	Virulent
AK208	Hope, AK	Cotton	-	Virulent
CKP	Pullman, WA	Chickpea	+	Virulent
AK89-1	Clarkedale, AK	Cotton	-	Virulent
BK28R	Clarkedale, AK	Cotton	-	Avirulent
BK101	Monterey County, CA	Cyclamen	+	Avirulent
BK102	Kings County, CA	Cotton	-	Avirulent
WASH	Bellingham, WA	Carrot	+	Avirulent
FL/W	Dade County, Florida	Carrot	-	Avirulent

* Bottacin *et al.*, (1994). The presence of dsRNA was confirmed by insensitivity to both DNAase and RNAase at high ionic strength and sensitivity to RNAase at low ionic strength. CF1 cellulose was also used to isolate dsRNA.

Table 2. Esterase activity in supernatants representing successive washes by centrifugation of spore suspensions of *Chalara elegans* from 14-day old cultures grown on V8 agar

Number Of Washes	Esterase Activity (virulent isolates)			Esterase Activity (avirulent isolates)		
	NC49A Nitrophenol (μmol/ml/min)	AK89-1 Nitrophenol (μmol/ml/min)	AK208 Nitrophenol (μmol/ml/min)	BK102 Nitrophenol (μmol/ml/min)	BK101 Nitrophenol (μmol/ml/min)	BK28R Nitrophenol (μmol/ml/min)
1	0.34	0.33	0.40	0.00	0.00	0.00
2	0.02	0.04	0.04	0.00	0.00	0.00
3	0.01	0.02	0.03	0.00	0.00	0.00
4	0.00	0.01	0.02	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00

C. elegans (CKP and BK28) started to release esterase after contact with plastic wrap for 40-45 minutes (Figure 4.). Avirulent isolates (BK28R and BK101) did not release detectable levels of esterase activity during the 120 minutes of incubation.

The effect of temperature on production of esterase activity by spores of *Chalara elegans*

Spores of virulent isolate BK28 of *C. elegans* released more esterase when incubated at 15° C and 24° C than when incubated at 5° C (Figure 5.).

The results of Tukey test (Figure 5.) showed that the difference in esterase release between spores incubated at either 15°C or 24°C and spores incubated at 5°C were significant. However, there was no significant difference in esterase release between spores incubated at 15° C and 24°C. Avirulent isolate BK28R of *C. elegans* did not release esterase during 45 minutes incubation any of these temperatures.

DISCUSSION

C. elegans, a fungal pathogen that causes black root rot on a broad range of hosts, has the ability to infect and cause lesions on both roots and

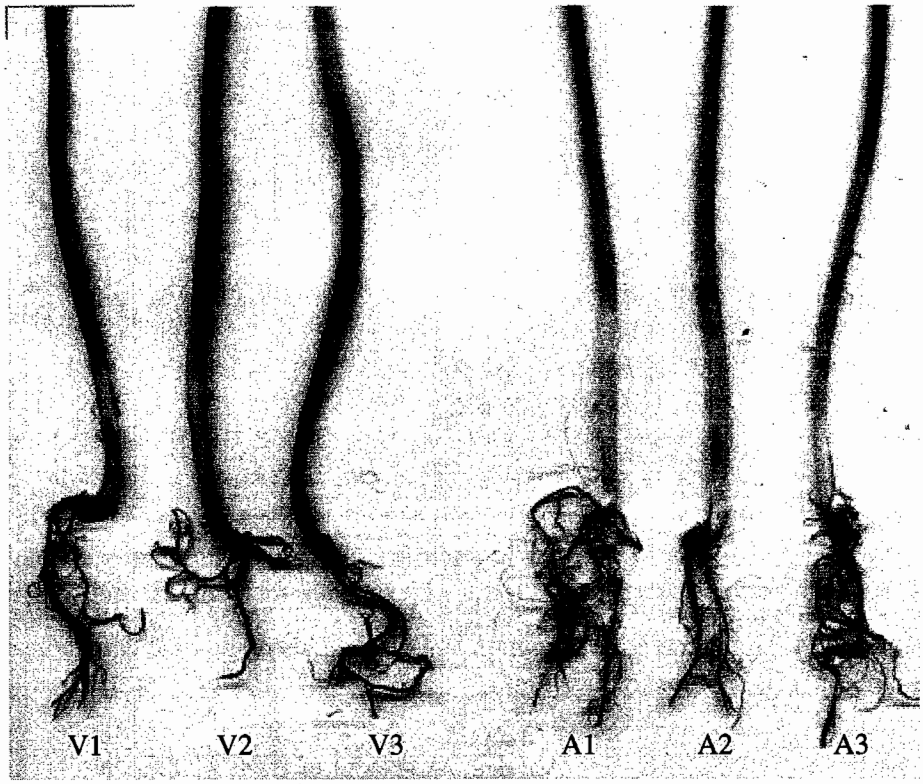

Figure 1. Symptoms on hypocotyls and roots of bean seedlings infected by virulent isolates (V1=BC 94PA; V2=BK28; V3=AK208) and avirulent isolates (A1=WASH; A2=BK28R; A3=BK101) of *Chalara elegans*.

hypocotyls of bean seedlings. A set of 12 isolates of *C. elegans* that differ in terms of geographic and plant host origin was found to differ qualitatively in their virulence on bean: seven isolates were virulent and five isolates were avirulent. Published information about the basis of virulence difference by isolates of *C. elegans* is lacking. However, failure to penetrate plant surface appeared to be a key factor that dis-tinguished avirulent isolates of *C. elegans* on beans (Sualang, 1997).

The results of this research showed that washed ungerminated spores of virulent isolates of *C. elegans* released esterase during 45 minutes of incubation on plastic wrap overlaid on V8 agar. Nicholson *et al.*, (1988) described a similar response when conidia of *Erisyphe graminis*, a fungal pathogen of wheat, was inoculated on cellophane and leaf surfaces and they suggested that this response was stimulated by a nonspecific contact stimulus. Esterase has also been reported to be released from ungerminated spores of *Uromyces viciae-fabae* and this phenomenon has been shown to be related to adhesion of spores to the leaf surface (Deising *et al.*, 1992). The erosion of leaf cuticle by esterase–rich conidial exudate from *E. graminis* (Kunoh *et al.*, 1990) indicated another possible function of esterase released by spores. Some researchers have used the degradation of cuticle as evidence to prove the release of hydrolytic enzymes from spores that can degrade cuticle (Hau & Rush, 1982; Staub *et al.*, 1974).

The fact that virulent but not avirulent isolates of *C. elegans* released esterase after incubation for 45 minutes on plastic wrap suggests that hydrolytic enzymes may be important in the penetration process for *C. elegans*. Contact between spores of virulent isolates and solid surfaces may stimulate the production of esterase. Contact stimulus has been considered as one of the causes of some fungal responses during host recognition (Wynn, 1981). Whether the nonspecific esterase released by the virulent isolates of *C. elegans* is cutinase is still in question. In these experiments, nonspecific esterase activity was obtained by using *p*-nitrophenylbutyrate as a model of substrate. Even though *p*-nitrophenylbutyrate has also been widely used as a suitable model substrate to assay cutinase activity (Kolattukudy *et al.*, 1981), conclusive proof for the release of cutinase by virulent isolates of *C. elegans* cannot be obtained using this model

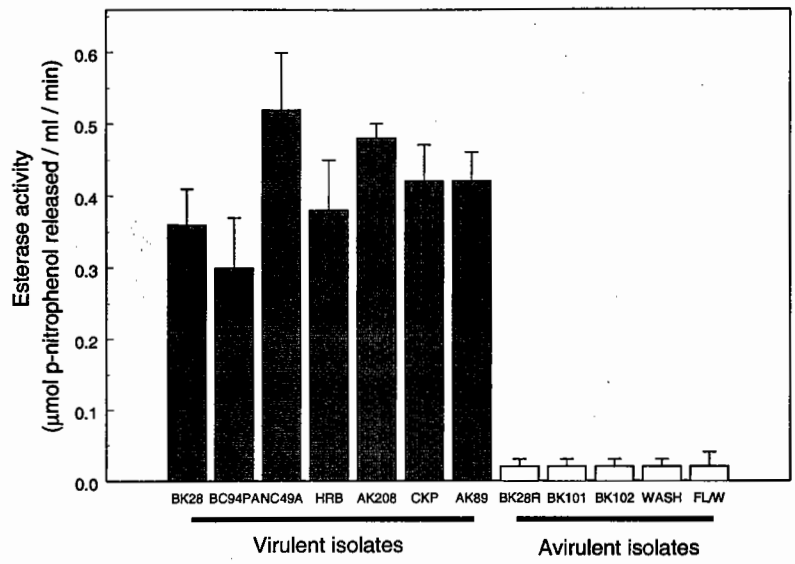

Figure 2. Histogram of esterase activity from unwashed and ungerminated spores of *Chalara elegans* obtained from 14-day-old cultures grown on V8 juice agar. The vertical bars indicate standard deviation of the mean of three replications

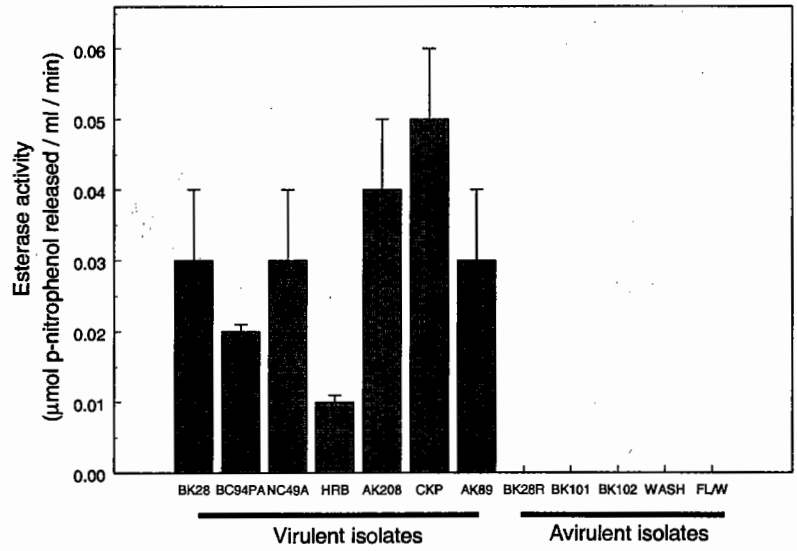

Figure 3. Histogram of esterase activity from washed and ungerminated spores of *Chalara elegans* after incubation on plastic wrap overlaid on V8 juice agar for 45 minutes. The vertical bars indicate the standard deviation of the mean of three replications.

substrate. According to Koller (1991), to prove the identity of cutinase activity, analysis of ^{14}C-cutin hydrolysis and the identification of hydrolysis products should be carried out.

It's found that washed spores of isolate BK28 started to release esterase 45 minutes after inoculation on plastic wrap and isolate CKP started to release esterase 5 minutes earlier than isolate BK28. Two hours after inoculation, esterase released by isolates BK28 and CKP was dramatically decreased. This fact suggests that the esterase released by isolates BK28 and CKP was unstable. Low temperature appears to decrease the ability of isolate BK28 and CKP to release esterase.

While growing on V8 juice agar, virulent isolates produced much more esterase than avirulent isolates. This observation again indicates that virulent and avirulent isolates of *C. elegans*

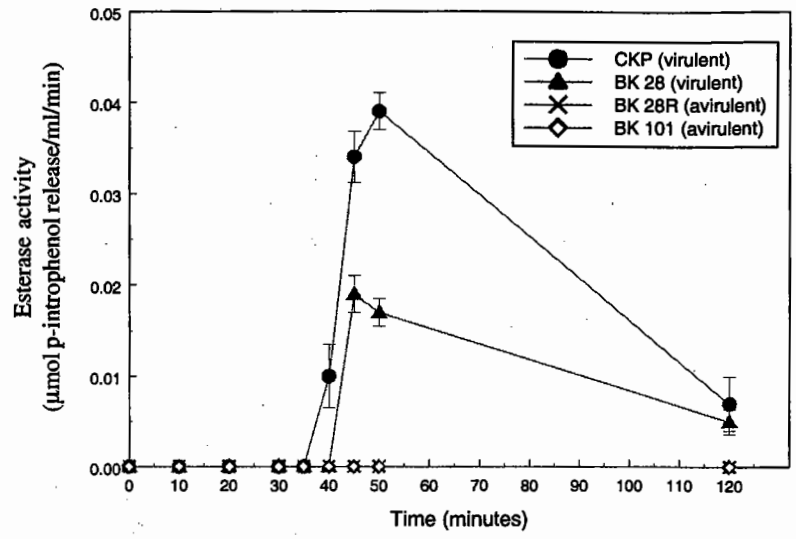

Figure 4. Graph of esterase activity from ungerminated washed spores of *Chalara elegans* as a function of time of incubation on plastic wrap on room temperature. The vertical bars indicate standard deviation of the mean of three replications

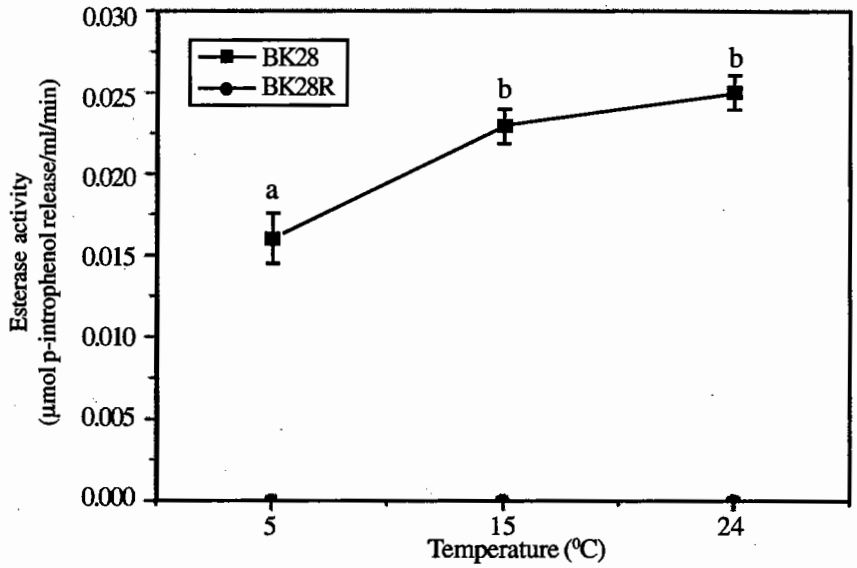

Figure 5. Graph of esterase activity from ungerminated washed spores of *Chalara elegans* (BK28, virulent isolate and BK28R, avirulent isolate) after incubation on plastic wrap at different temperatures for 45 minutes. The vertical bars indicate the standard deviation of the mean of three replications. The letters indicate the results of Tukey test; Same letters indicate no significant difference between the means; Different letters indicate a significant difference between the means.

differ in their ability to produce esterase. Whether the esterase activity released by spores *of C. elegans* while they were growing in agar, and the esterase activity released after contact with solid surface are the results of the same esterase enzymes is as yet unknown. It has been reported that some pathogenic fungi release cutinase in the presence of cutin in growth medium (Kolattukudy & Koller, 1983). Since V8 juice agar may have contained cutin, it may reasonable to suggest that esterase released by *C. elegans* in the medium might be cutinase. The fact that *p*-nitrophenyl butyrate was used as substrate for esterase assays in this experiment, and also has been used as a model of substrate for cutinase assays, indicates that esterase released by *C. elegans* might be cutinase.

The presence of double-stranded RNA fragments (dsRNAs) in plant pathogenic fungi has been

reported (Boland, 1992; Bottacin *et al.*, 1994; Tooley, 1989) and can cause either no measurable effects or alteration of fungal virulence. In the rust fungi, the presence of dsRNAs has been reported to have no effect on fungal virulence (Zhang *et al.*, 1994). However, the presence of dsRNAs has been associated with reduced virulence in *C. elegans* (Bottacin *et al.*, 1994). Among isolates of *C. elegans* used in these experiments, six isolates contain dsRNA and five isolates contain no dsRNA (Bottacin *et al.*, 1994). The ability to produce nonspecific esterase activity seems to have no correlation with the presence of dsRNA. Isolates AK89-1 and AK 208 with no dsRNA produced esterase activity both in culture on V8 juice agar and 45 minutes incubation on plastic wrap, whereas two other isolates, WASH and BK 101 with dsRNA did not release esterase after contact with plastic wrap.

The results of this research show correlation of esterase activity with virulence of *C. elegans* isolates on bean. However, the ability of virulent isolates of *C. elegans* to produce esterase after inoculation on plastic wrap surfaces does not necessarily indicate the involvement of this enzyme in infection process of this fungus on bean. Therefore, further investigations are required to provide direct evidence for a causal role of esterase activity in virulence of this fungus.

CONCLUSIONS

The results showed that after incubation on plastic wrap for 45 minutes, the virulent isolates but not avirulent isolates released esterase. This correlation suggests that esterase may be involved in penetration process of *C. elegans*. Both virulent and avirulent isolates released extracellular esterase activity while they were growing on V8 juice agar However, the amount of esterase activity released by virulent isolates in V8 juice agar was much higher than that released by avirulent isolates. Whether esterase released by *C. elegans* on plastic wrap and on V8 juice agar are the same class or different class of esterase is unknown. Virulent isolates started to release esterase 40 to 45 minutes after incubation on plastic wrap surfaces. Esterase activity released by virulent isolate was decreased by low temperature (5°C).

FUTURE DIRECTIONS

Penetration by *C. elegans* with appresorium formation is rarely found. Therefore, hydrolytic enzymes are suggested to have greater involvement in the penetration process of *C. elegans* than do mechanical forces. There are several observations that suggest that cutinase, a serine esterase, is involved in the penetration of host surfaces during plant infection. Cutinase is a hydrolytic enzyme that is suggested to be responsible for the disruption of cutin, the polymeric component of plant cuticle. The results of these experiments show that esterase activity was released by virulent isolates but not by avirulent isolates after incubation on plastic wrap. The results also showed that that esterase was released by both virulent isolates and avirulent isolates of *C. elegans* on V8 juice agar. Since cutinase is an esterase, it will be important to find out whether the esterase released by *C. elegans* isolates on either plastic wrap or growth agar is cutinase using radioactive-cutin as a substrate.

In this observation, virulent isolates of *C. elegans* were found to release esterase after incubation on artificial surfaces. This finding does not necessarily indicate the involvement of this enzyme during plant penetration. To prove the involvement of esterase in penetration process of virulent isolates of *C. elegans* into on bean tissues, the secretion of esterase by the penetrating fungus at the site of infection must be shown. Therefore, further investigation is required to determine whether virulent isolates penetrating into intact surfaces of bean release esterase. The use of esterase inhibitor will also be necessary to prove the involvement of esterase in penetration of *C. elegans*. If esterase is crucial for penetration process, inhibition of esterase activity should prevent penetration process and therefore infection process of *C. elegans*.

Decreased virulence of *C. elegans* has been found to correlate with the presence of dsRNA fragments in this fungus (Bottacin *et al.*, 1994). However, the mechanism of dsRNAs action in the reduction of virulence of *C. elegans* is not known yet. Further information about functions encoded by dsRNAs will be important to understand the effect of dsRNAs on the interaction of *C. elegans* with its hosts.

BK 28R (avirulent isolate) has been found not to be able to penetrate bean surfaces. Further investigation is required to find out more about what

possible defect in this isolate.

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